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SOLUBLE CELL-SURFACE DIMERIC PROTEINS

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Solubilized poly (sub-unit) surface membrane proteins are obtained by modifying genes with a signal for phosphatidyl anchored proteins. By expressing the modified proteins in an appropriate host, the assembled surface membrane protein may be obtained in a form where it may be released from the membrane and used for a variety of purposes.

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SOLUBLE CELL-SURFACE DIMERIC PROTEINS

INTRODUCTION

Technical Field

The field of this invention is preparation of soluble cell surface poly(sub-unit) proteins as soluble entities.

BACKGROUND OF THE INVENTION

Cells are continuously interacting with their environment, receiving cues concerning the state of the environment and signals associated with the response of the cell to the environment. In a multicellular host, the cells interact with the environment, influencing the environment by secreting various compositions, removing compositions from the environment, and in some instances moving away from or toward a particular environment. Many different mechanisms are used for the cell to receive a signal from the environment and translate that signal into a response.

In many cases, the cell relies upon a surface membrane protein, which may be a single chain or a plurality of chains or in some instances, for the purposes of transducing a signal, two or more independent proteins may be associated. Proteins which respond to hormones, cytokines, glucocorticoid steroids, antigens, and other surface membrane receptors, are only of the few of the surface membrane proteins present on a mammalian cell.

For many purposes, it would be useful to have the surface membrane protein in a soluble form free of the cell membrane or microsome. In this way, the nature of binding of the protein to its ligand or other protein could be studied. In addition, the soluble form of the surface membrane protein may be used prophylactically or therapeutically, as an agonist or antagonist, to induce

or inhibit the transduction of a signal.

A substantial proportion of the surface membrane proteins are bound to the surface by means of a hydrophobic domain present in the protein. By having a combination of a signal sequence at the N-Terminus and a transmembrane integrator sequence or domain as an internal portion of the protein, the transport of the membrane protein through the membrane is arrested at the transmembrane integrator sequence. Furthermore, for many surface membrane proteins, the protein is poly(sub-unit), having two or more sub-units, which may be covalently or non-covalently bound together. The association of the sub-units in the cell and their transport to the surface is not understood. Therefore, in making changes in the protein, in order to provide it in soluble form, it is not at all clear that modifications of the protein will provide for proper folding and proper association and processing, if the protein is retained intracellularly or is secreted. It is therefore of substantial interest to find methods for producing proteins having a plurality of sub-units in a form where they are properly folded and associated and may be readily isolated in soluble form.

Relevant Literature

T cell Receptor (TCR) heterodimers are assembled with the CD3 polypeptide to form complexes of at least seven polypeptides before appearing in the surface (Minamiel et al., Proc. Natl. Acad. Sci. USA (1987) 84:2688). TCR (V)-Ig(C) hybrids expressed in myeloma cell lines have been described (Gascoigne et al., ibid (1987) 84:2936). Only the Vα (TCR) C_4(Ig) chimeras were assembled or secreted. Lipid-linked surface proteins form a significant class of surface membrane proteins (Ferguson and Williams, Ann. Rev. Biochem. (1988) 57:285). Caras et al., Science (1987) 238:1280, has shown that the carboxy-terminal thirty-seven imino acids

of decay accelerating factor could serve as the signal sequence for the lipid-linked expression of a herpes simplex virus membrane protein. The carboxyl terminus of human placental alkaline phosphatase, a homodimer, has been shown to be a lipid-linked molecule (Kan et al., Proc. Natl. Acad. Sci. USA (1985) 82:8715). Attempts to use TCR-Ig chimeras have been reported by Traunecker et al., Immunol. Today (1989) 10:29.

SUMMARY OF THE INVENTION

Methods are provided for producing in soluble form multi(sub-unit) surface membrane proteins. The resulting proteins may be used in the study of their interactions with their ligands, other surface membrane proteins, or as agonists or antagonists for the interaction of the naturally occurring surface membrane protein. The technology is exemplified with the T cell receptor.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

The subject invention concerns solubilizing surface membrane proteins of the mammalian hosts. The surface membrane proteins are characterized by being bound to the membrane by a hydrophobic sequence, normally having charged or polar amino acids at its boundaries. The proteins will also have a plurality of sub-units, where one or more of the sub-units will comprise the hydro-phobic sequence known as a transmembrane integrator sequence or domain. In addition, the surface membrane protein will have translocation signals, such as signal sequences at the N-terminus of the protein. The signal sequences will direct the translocation of the sub-units with processing from the Golgi apparatus through the membrane, where the signal sequence will normally be cleaved during the processing to produce a protein sub-unit free of the signal sequence.

The proteins of interest will generally have from about 2 to 8, usually 2 to 6, more usually 2 to 4, sub-units. Desirably, each of the sub-units will have not more than 2, usually not more than 1 transmembrane integrator sequence, so that the sub-unit passes through the surface membrane only once. The size of the sub-units is not critical to this invention and may vary from at least about 10kDal (kiloDaltons) and is usually not more than 1000kDal, usually not more than 600kDal, more usually not more than 200kDal. In many cases, the sub-unit protein may be associated with other proteins at the membrane surface, which proteins may be involved with the formation and transport of the multi-sub-unit proteins.

A wide variety of surface membrane proteins fulfill the above requirements, particularly receptors, enzymes, and the like. These proteins include T cell receptors, surface immunoglobulins, major histocompatibility complex antigens, both Class I and II, hormone receptors, G proteins, etc.

In order to solubilize these proteins, they will be modified by removing at least in part the transintegrator membrane sequence of each subunit and providing for a signal which results in the attachment of a lipid to the sub-unit and transport of the sub-unit to the surface membrane where it is non-covalently bound to the surface membrane.

The particular signal sequence may be from any convenient source which is functional in the expression host, so that it may be endogenous or exogenous to the expression host or common or foreign to the source of the surface membrane protein. The signal sequence may be associated with a surface membrane protein which has a single unit or a multiplicity of sub-units, preferably from a protein which has a multiplicity of sub-units. Illustrative signal sequences are derived from such proteins as decay accelerating factor, placental

alkaline phosphatase, and the like. The sequence providing for lipid attachment will generally include a sequence which has from about 10 to 50, more usually from about 15 to 30 amino acids which are cleaved from the carboxyl terminus of the sub-unit precursor and an ethanolamine-carbohydrate-phosphatidylinositol linked to the new carboxyl terminus. Once the precursor has been processed in this way, it is then transferred to the cell surface.

The cells may be isolated containing the modified sub-units anchored to the surface membrane by means of lipid and the various portions of the anchor removed individually or together. Pronase cleaves at the penultimate peptide of the amino acid bonded to the C-terminal amino acid which is bonded to ethanolamine. Nitrous acid may be used to cleave a glycosidic linkage between an amino sugar, glucosamine and another sugar inositol. Phosphatidylinositol-phospholipase C(PI-PLC) may be used to cleave at the phosphate linkage between the diacylglycerol and inositol phosphate.

Usually, the phosphatidyl inositol anchor signal will only be partially removed, leaving from about 10 to 30 amino acids remaining at the carboxyl terminus of the sub-unit of interest. The sequence provides a convenient tag for isolation and binding. Where this additional sequence does not interfere with the purpose of the membrane protein, it may be left with the protein. Alternatively, by using carboxypeptidase, some or all of the additional amino acids may be removed. Various techniques exist for removing amino acids from the C- terminus of a protein individually. While this is not convenient, in some individual cases this may prove to be expedient. Preferably, a sequence may be introduced between the phosphatidyl inositol anchor sequence and the sub-unit sequence which provides a signal for a peptidase enzyme.

A wide variety of sequences are available which

are specifically recognized by enzymes, where the sequence is not encountered in the sub-unit sequence of interest and the enzyme is not expected to be encountered in the expression host. For example, the peptidase signal sequence of the alpha- or a-protein of yeast, the Kex enzyme, or a sequence recognized by any other convenient enzyme, may be used to provide for removal of the peptidase signal and the phosphatidyl inositol anchor sequence from the sub-unit.

In providing for expression of the individual sub-units, constructs will be prepared, where the sub-unit is modified, as appropriate, by removal of at least a portion of the transmembrane integrator sequence and the cytoplasmic sequence. The sequences will be replaced by the phosphatidyl inositol anchor sequence from the appropriate protein. The exchange may be achieved in a variety of ways. The sub-unit gene may be cloned and by employing <u>in vitro</u> mutagenesis or primer repair, the transmembrane integrator and cytoplasmic sequences may be removed and in the case of <u>in vitro</u> mutagenesis, replaced with the phosphatidyl inositol anchor sequence. Depending upon the size of the gene, one may use the polymerase chain reaction, where the primers comprise the phosphatidyl inositol anchor region and a sequence of at least 10 nucleotides, preferably at least about 18 nucleotides, which are complementary to the sequence of the sub-unit gene 5' prime of the transmembrane integrator sequence. Other manipulations may include restriction at a site proximal, preferably 5'-proximal, to the transmembrane integrator sequence and then insertion of a sequence comprising the phosphatidyl inositol anchor sequence at the 3' terminus of the truncated sub-unit gene. After ligation, the chimeric construct may then be cloned, the DNA isolated and used for expression. Of course, the sequence which includes the phosphatidyl inositol anchor sequence will also include a peptidase signal sequence at its 5'-

terminus for cleavage at that site by the peptidase and removal of the extraneous amino acids.

The gene for the sub-unit may be the genomic gene or cDNA, preferably cDNA. The genomic gene, to the extent that it includes introns, will not be as manageable during the various constructs and manipulations. Where this is not a problem, the genomic gene may find preference in providing for a higher level of expression. Normally, the gene will be known and sequenced, so as to allow for ready manipulation, identification of restriction sites, and ease of introduction and removal from vectors. The sub-unit gene may be isolated, cloned in an appropriate cloning vector and then manipulated by various techniques as described above. See, Maniatis et al., A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. The transmembrane integrator and cytoplasmic sequences may be removed and replaced with the phosphatidyl inositol anchor sequence as obtained from an appropriate gene. By appropriate manipulations, overhangs or blunt ends may be provided for ligation of the two sequences in proper reading frame. The construct may then be cloned and analyzed by any convenient means, including restriction analysis, sequencing, hybridization, or the like. Once the desired construct has been prepared, it may then be used in an appropriate expression vector in an appropriate expression host.

A wide variety of hosts are available, but in view of the desirability for processing and assembling of the surface membrane protein, normally an appropriate mammalian host will be employed for expression. The vectors which are selected will normally include a marker for identification of those hosts into which the construct has been introduced, where the host may be identified by positive selection. Thus, the marker will usually be protection from a biocide, particularly an

antibiotic, e.g. G418, or in appropriate cases, where the gene provides prototrophy to an auxotrophic host. For the most part, the vectors will not be stable in the expression host, so that the gene will be integrated into the genome of the host. However, in some instances, it may be desirable to retain the construct as part of an episomal element. Viral replication systems may be employed, such as those of SV40, papilloma virus, adenovirus, etc., where the viruses have been attenuated, are capable of accommodating the construct, and are usually not lytic. Various constructs have been described in the literature, and need not be exemplified here.

Various techniques may be employed for introducing the construct into the host, such as calcium phosphate precipitated DNA, transfection, transduction, electroporation, fusion, etc. After the cells have been transformed with the construct, the cells may be grown on an appropriate selective medium for selection of those hosts containing the marker.

Various expression hosts have been developed for use and expression of mammalian proteins. These hosts include chinese hamster ovary cells, COS cells, mouse mammary kidney cells, HeLa cells, and the like. The cells may be grown in an appropriate medium, where the desired proteins are translocated to the membrane. The host cells may then be harvested, lysed, and the microsomes treated with pronase and the resulting proteins isolated. The proteins may be further processed to remove all or part of the lipid anchor signal sequence. The desired surface membrane protein may be purified by affinity chromatography, electrophoresis, HPLC, or the like. Once the protein has been isolated, it may be used in a variety of ways.

As already indicated, the soluble proteins may be used in the study of the interaction with other surface membrane proteins or ligands. For example, the

soluble T cell receptor may be used in studying the interaction with major histocompatibility complex antigens, Class I or Class II, or the like. Alternatively, they may provide for cells with varying levels of the surface membrane protein bound to the surface, by varying the periods of time that the cells are contacted with pronase, phospholipase C, nitrous acid, or the like. In this manner, the effect of the number of the surface membrane protein molecules on the surface in relation to other surface membrane proteins may be investigated.

Besides using the soluble proteins to study various interactions and gain an understanding of how the surface membrane proteins fulfill its function, the surface membrane proteins may also be used in culture and in vivo in various hosts. The soluble surface membrane proteins may be used as agonists or antagonists. For example, the soluble T cell receptor may be used to prevent interaction between T cells and other cells. When the other cells are B lymphocytes, production of antibodies may be prevented by preventing the binding between the T cell receptor and the MHC antigen of the B lymphocyte. With other proteins, they may be used to identify ligands, binding to other surface membrane proteins, as agonists or antagonists, affinity columns, or the like.

The subject soluble surface membrane proteins may be formulated in any convenient medium for administration to mammalian host. The proteins may be administered in buffered or unbuffered solutions, such as saline, phosphate buffered saline, phosphate, aqueous ethanol, or the like. The concentration of the protein will generally vary from about 0.001 to 5 mg/ml. Dosage will vary widely depending upon the particular condition being treated, the method of administration, the frequency of administration, and the like. The amount administered would generally range from about 1pg to

5mg/kg host.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

The PI-anchored TCRs were constructed by fusing the PI-anchor signals (DAF or HPAP) to the fifth amino acid residue of the TCR located 3' to the last cysteine residue before the transmembrane domain. Full length cDNAs encoding the 2B4 TCR α or β chains (Chien et al., Nature (1984) 309,322; Berkin et al., Nature (1985) 317,430) were inserted 5' to the DNA segment coding for the last 37 amino acids of DAF (residues 311-347) (Caras et al., Nature (1987) 325,545) or the last 47 amino acids of HPAP (residues 467-513) (Kam et al., PNAS USA (1985) 82:8175); Micanovic et al., ibid (1988)85:1398) cloned in plasmid bluescript pSK-. The in frame joining between the TCR and the PI-anchor signal was performed by oligonucleotide directed in vitro deletional mutagenesis (Kunkel et al., Methods in Enzymology (1987) 154,367; Amersham Handbook RPN 2322 (1986)). In all cases, synthetic 32-oligomers that span the junctional point with 17 nucleotides complementary to the DAF or HPAP sequences and 15 to the TCR sequences were used as primers. After in vitro mutagenesis, only the first 227 and 265 amino acid residues of the TCR α β chains respectively were retained in the chimeric fusion protein. The truncated α and β chains were fused to residue 311 of DAF and/or to residue 484(HPAP-L) or 495(HPAP-S) of HPAP. The recombinant DNAs were sequenced after mutagenesis. The TCR α and β DAF/HPAP genes were then inserted in an expression vector (pSRα1 Neo is a derivative of pcDL-SRα296 (Takeke et al., Mol. Cell Bio. (1988) 8:466) to which a polylinker containing the restriction sites of 5'XhoI/XbaI/SαlI/NaeI/EcoRI/EcoRV/HindIII/ClaI is placed between the SRα promoter and polyadenylation site for

convenient cDNA cloning. In addition, a neomycin resistant gene under the control of the SV40 early promoter is inserted between the short arm between the ampicillin gene and the SRα promoter containing the neomycin gene generating constructs respectively called αβDAF, αβHPAP-L and αβHPAP-S. Both α and β fusion genes were under the control of the SRα1 promoter.

The following are the sequences for the PI anchor signals:

LAPPAGTTD AAHPGRSVVPALLPLLAGTLLLLETATAP HPAP-S

CLEPYTACDLAPPAGTTD AAHPGRSVVPALLPLLAGTLLLLETATAP HPAP-L

PMKGSGTTSGTTRLLSGMTC FTLTGLLGTLVTMGLLT DAF

Comparison of CHO transfectants co-expressing TCR α and β chains on the cell surface with various PI-anchoring signals.

CHO cells were transfected with αβDAF, αβHPAP-L or αβHPAP-S constructs using electroporation. The G418 resistant colonies were pooled and analyzed for surface expression of the PI-anchored TCR α and β chains. Cells were sequentially stained at 4°C for 1 hour in phenol red free RPMI+ 5% fetal calf serum with the hamster anti-mouse KJ25 antibody (anti-Vβ3) (2 μg/ml) and then with the biotinylated A2B4.2 (anti-Vα11). FITC conjugated goat anti-hamster immunoglobulin and streptavidin were used as second receptors, respectively. After staining, the cells were subjected to FACS analysis and the brightest 5% double positive cells co-expressing TCR α and β fusion proteins were sorted. After two weeks in culture, the pooled cells were sorted a second time for the highest 5% double expressors and grown as massive culture for further studies. The clones were analyzed again a few weeks later for the stability for surface expression. The data were plotted as log fluorescent intensity in an arbitrary unit. After long term culture, cells

transfected with αβDAF and αβHPAP-L both contain a significant population of dull double positive cells as well as bright double positive cells. The αβHPAP-S transfected cells which seemed to have stably integrated the TCR fusion genes were used for subsequent studies.

Blocking of KJ25 antibody binding to the PI-anchored TCR by A2B4.2.

$5x10^5$ Jurkat β- cells expressing a functional 2B4 αβ TCR after transfection with wild type 2B4α and β genes and CHO transfected with the αβHPAP-S construct were pretreated with or without antibody A2B4.2 (20/μg/ml) and then sequentially incubated with antibody KJ25 (Vμg/ml) and a FITC conjugated goat anti-hamster immunoglobulin. Cells were analyzed on the FACS after staining. The data were plotted as log fluorescent intensity in arbitrary units. The presence of A2B4.2 abolishes the subsequent binding of KJ 25 on both Jurkat β- cells and CHO αβHPAP-S transfected cells. The Jurkat β- population is non-clonal which explains the presence of negative cells (50%) after staining with KJ 25. CHO cells have a higher fluorescence background than Jurkat cells as indicated by propidium staining.

Purification of a soluble TCR heterodimer.

A. Summary of soluble TCR purification scheme.

$2x10^8$ CHO αβHPAP-S transfected cells were seeded into the Cell PharmI Bioreactor (CD Medical). After one week of culture (10^{10} cells present in the Bioreactor), the cells were treated with 10 units of PI-PLC at 37 degrees C for three hours (Tse et al., Science (1985) 230,1003; Hon and Kincarde, Nature (1985) 318,62). The chimeric TCR was recovered in 200 ml of growth media pulsed into the Bioreactor. The supernatant was filtered, adjusted to pH 8.0 and passed over an A2B4.2 cyanogen bromide affinity column (1 ml) pre-equilibrated with 3 volumes of 0.1M phosphate/0.15M NaCl, pH 8.0

buffer. The column was then washed with 3 volumes of the same buffer. The protein was eluted with 3 volumes of 0.1M NaOAc/0.15M NaCl, pH 3.5 buffer in 0.5 ml fractions and instantly neutralized with a solution of 2M Tris-HCl pH 8.5 (1/10 volume added). The first four fractions which contain most of the soluble TCR were pooled and applied to a KJ 25 affinity column. The protein was then eluted at pH 4.0 with the buffer described above.

B. Detection of soluble TCR on silver stained polyacrylamide gels.

Ten μl of each fraction eluted form the A2B4.2 and KJ 25 columns were electrophoresed on a 12.5% SDS-polyacrylamide gel under reducing or non-reducing conditions. The protein gel was stained with silver nitrate (Morrisey, Anal. Biochem. (1981) 117,307). The protein eluted from the A2B4.2 column contains both monomers (α, 41 Kd) and dimers (α2 and αβ, 70 Kd). Under reducing conditions, the dimers were reduced to the size of monomers. Both 2B4 α and β chains have the same molecular weight (41 Kd) and therefore cannot be distinguished from each other on the gel. The αβ heterodimer is separated from α monomer and α2 dimer after passage over the KJ 25 column, as confirmed in C.

C. Immunoprecipitation of the purified soluble TCR.

One μg of purified soluble TCR eluted from either the A2B4.2 or the KJ 25 column was iodinated with Na ^{125}I to the specific activity of $6x10^7$ cpm/μg using Enzymobeads (Bio-rad). $2x10^6$ cpm of iodinated TCR was precipitated with either A2B4.2, KJ 25 or 14-4-4s antibody, respectively. Antibody 14-4-4s which recognizes the Eα chain of MHC class II is used as a negative control. The immunoprecipitates were subjected to electrophoresis on a 1.25% SDS-polyacrylamide gel

under reducing (C) and non-reducing (D) conditions. The gels were dried and submitted to autoradiography. Again, a band corresponding to a 70 Kd protein can be visualized on the autoradiogram. The intensity of the signal in lane 1 is more intense than in lane 2 (A2B4.2 antibody lanes 1-3) while both signals in lanes 4 and 5 (KJ 25 antibody, lanes 4-6) have identical intensities. This result indicates the presence of not only heterodimers ($\alpha\beta$) but also homodimers ($\alpha 2$) after elution from the A2B4.2 column (lane 1). The heterodimers are eliminated after passage of the fractions over the KJ 25 column. The ratio of α versus β molecules then becomes identical which proves the presence of heterodimers exclusively after passage of the fractions over both columns.

From the results of silver staining and immuno-precipitation, it is estimated that the $\alpha\beta$ 2 heterodimer represents approximately 60% of the total PI-PLC cleaved products or 80% of the dimers.

To verify that the $\alpha\beta$ 2 heterodimer is the product from the PI-PLC Cleavage, the soluble TCR was immuno- precipitated with two rabbit antisera anti-CRD 1 and anti-CRD 2, which recognize the phosphoglycan epitope exposed after PI-PLC treatment. The two antisera showed lower affinity for $\alpha\beta$ 2 heterodimer than α 2 homodimer. This is consistent with observations with other TCR systems.

Following the above described procedures with some modifications, as indicated, the PI-anchored TCRs were constructed by fusing the PI-anchor signals (DAF or HPAP) to the fifth amino acid residue of the TCR located 3' to the last cysteine residue before the transmembrane domain. Full length cDNAs encoding the 2B4 TCR α or β chains (Chien et al., Nature (1984) 309, 322; Becker et al., Nature (1985) 317, 430) were inserted 5' to the DNA segment coding for the last 37 amino acids of DAF (residues 311-347) (Caras et al., Nature (1987) 325,

545) or the last 38 or 47 amino acids of HPAP (residues 476-513 and 467-513, respectively) (Kam et al., PNAS USA (1985) 82, 8715); Micanovic, ibid (1988) 85, 1398 cloned in plasmid bluescript pSK-(Stratagene). The in-frame joining between the TCR and the PI-anchor signal was performed by oligonucleotide directed in vitro deletional mutagenesis (Kunkel et al., Meth. in Enzymol. (1987) 154, 367). After mutagenesis, the first 227 and 265 amino acid residues of the TCR α and β chains respectively were retained in the chimeric fusion protein. The truncated α and β chains were fused to residue 311 of DAF and/or to residue 484 (HPAP-L) or 495 (HPAP-S) of HPAP.

A comparison was made of CHO transfectants coexpressing TCR α and β chains on the cell surface with various PI-anchoring signals. CHO cells were transfected with αβDAF, αβHPAP-L or αβHPAP-S constructs (inserted into pBJ1-Neo. (The cDNA expression vector, pBJ1-Neo, is a derivative of pcDL-SRα296 (Takeke et al., Mol. Cell Biol. (1988) 8:466 in which a XhoI fragment located between the SRα(HTLV-1) promoter and the SV40 polyadenylation site has been replaced for convenient cDNA cloning by a polylinker that contains the following restriction sites: 5' XhoI-XbaI-Sfi-I-NotI-EcoRI-EcoRV-HindIII-ClaI-3'. In addition a neomycin gene (resistance to antibiotic G418) was inserted in between the ampicillin resistance gene and the SRα promoter)) by electroporation (Chu et al., Nucleic Acids Res. (1987) 15:1311). The G418 resistant colonies were pooled and analysed for surface expression of the PI-anchored TCR α and β chains. Cells were sequentially stained at 4°C for 1 hour in phenol red free RPMI + 5% fetal calf serum with KJ25 (anti-Vβ3) and biotinylated A2B4.2 (anti Vα). Fluorescein isothiocyanate (FITC) conjugated goat anti-hamster immunoglobulins and streptavidin-PE were used as secondary antibodies.

$5X10^5$ Jurkat β-cells expressing a functional 2B4

αβ TCR after transfection with 2B4 α and β genes (Sarto and Germain, Nature (1987), 329, 256) (A and B) and CHO cells transfected with the αβHPAP-S construct (C and D) were pretreated with (B and D) or without (A and C) A2B4.2 (20 μg/ml) and then KJ25 (2 μg/ml) FITC-conjugated goat anti-hamster immunoglobulins (A2B4.2 is a mouse antibody and KJ25 is from a hamster, such that only the latter will stain with the FITC reagent). Cells were analysed by flow cytometry after staining. The data were plotted as log fluorescent intensity in arbitrary units. Approximately half of the 2B4 αβ transfected Jurkat cells are negative for TCR, and half are positive accounting for the two populations that were visible in the profile. The Y axis shows propidium iodine staining, a measure of cell viability, while the X-axis shows the degree of KJ25 flourescence.

When 2B4 Jurkat cells are first treated with the antibody to Va and then with anti-Vβ3, staining with the latter is completely inhibited. The same inhibition effect can be observed after pretreatment of αβHPAP-S, αβDAF and αβDAP-L transfectants. KJ25 staining of the CHO cells is inhibited by 90% after pre-incubation with A2B4.2, supporting the PI-anchored TCR β-chains being expressed on the cell surface as heterodimers with the α chain and in a conformation indistinguishable from the native structure, even in the absence of CD3.

The TCR heterodimer was purified as follows: $2x10^8$ αβHPAP-S transfected CHO cells were seeded into a Cell Pharm I Bioreactor (CD Medical). After two weeks of culture ($2x10^{10}$ cells present in the Bioreactor), the cells were treated with approximately 10 units of PI-PLC at 37°C for three hours (Becker et al., Nature (1985), 317, 430). The chimeric TCR was recovered in 250 ml of growth media pulsed into the Bioreactor. The supernatant was filtered, adjusted to pH 8.0 and passed over an A2B4.2 (cyanogen bromide coupled sepharose) affinity column (1 ml) pre-equilibrated with 0.1 M

phosphate/0.15M NaCl, pH 8.0 buffer. The column was then washed with the same buffer. The protein was eluted with 0.1M NaOAc/0.15M NaCl, pH 3.5 buffer in 0.5 ml fractions and neutralized immediately with 2M Tris-HCl pH 8.5 (1/10 volume added). The first four fractions which contain most of the soluble TCR were pooled and applied to a KJ25 affinity column. The protein was then eluted at pH 5.0 with 0.1M NaOAc/1M NaCl.

Sample eluates from the A2B4.2 (lanes 1, 3 and 4) or A2B4.2 + KJ25 (lanes 2, 5 and 6) columns were electrophoresed on a 10% SDS-polyacrylamide gel under reducing (lanes 1 and 2) and non-reducing (lanes 3-6) conditions. When run under non-reducing conditions, the samples were either not boiled (lanes 3 and 5) or boiled (lanes 4 and 6) before being loaded on the gel. The gel was stained with silver nitrate (Morrissey, Anal. Biochem. (1981), 117, 307). The fraction eluted from the A2B4.2 column, not boiled (lane 3) or boiled (lane 4) before being loaded on the gel, contains both monomers (α, 41 Kd) and dimers ($\alpha\alpha$ and $\alpha\beta$, 70 Kd). After subsequent passage of this fraction over the KJ25 column and elution at pH 5.0, only dimers are detectable (lane 5, sample not boiled) showing a separation of $\alpha\beta$ heterodimers from a monomers and aa dimers. If the sample is boiled (lane 6), low quantities of monomers become detectable. Under reducing conditions (lanes 1 and 2), dimers are reduced to the size of monomers.

The data demonstrate the presence of heterodimers and a small amount of dimeric molecules which are not disulfide-linked. Estimating from the silver stained gel indicates about 40% monomers and 60% dimers cleaved off the surface of the CHO cells of which 50% of the dimers are heterodimers (30% of the total).

It is evident from the above results, that in accordance with the subject invention, soluble surface membrane proteins having multiple sub-units can be

obtained with appropriate conformation. The epitopes exhibited by the proteins are bound by antibodies which recognize the naturally occurring surface membrane proteins. The surface membrane proteins may be used in a variety of ways, in studying the nature of their action with other proteins, for use as agonist or antagonists with the surface membrane protein, for screening compounds which bind to the surface membrane protein, and the like.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A soluble protein having at least two subunits and characterized by comprising the external portion of a surface membrane protein having not more than one transmembrane domain per subunit, being free of the transmembrane region and the cytoplasmic region and having the conformation of the naturally occurring surface membrane protein.

2. A soluble protein according to Claim 1, wherein said protein is obtained by substituting at least a portion of the transmembrane region with a signal for lipid attachment.

3. A soluble protein according to Claim 2, wherein said signal encodes for the attachment of a lipid containing sequence comprising a phosphatidyl inositol.

4. A soluble protein according to Claim 1 comprising a portion of the amino acid signal for attachment of a lipid.

5. A soluble protein according to Claim 1, free of amino acids of the signal for attachment of a lipid.

6. A soluble protein according to Claim 1, wherein said protein is a T cell receptor.

7. A soluble protein having at least two subunits and characterized by comprising the external portion of a surface membrane protein having not more than one transmembrane domain per subunit, being at least substantially free of the transmembrane region and the cytoplasmic region, having at least substantially the conformation of said surface membrane protein and comprising at the C-terminus a portion of the amino acid signal sequence encoding the signal for lipid attachment.

8. A soluble protein according to Claim 7, wherein said lipid attachment comprises phosphatidyl inositol.

9. A soluble protein according to Claim 8, wherein said lipid attachment comprises ethanolamine-

carbohydrate-phosphatidyl inositol.

10. A cell comprising the external portion of a surface membrane protein having at least two subunits, having substantially the same conformation of the natural surface membrane protein and bound to the surface membrane of said cell through lipid, wherein said surface membrane protein is naturally bound to said cell surface by a transmembrane domain.

11. A cell according to Claim 10, wherein said lipid is a phosphatidyl inositol.

12. A cell according to Claim 11, wherein said cell is a eukaryotic cell.

13. A cell comprising DNA encoding for the subunits of a surface membrane protein having at least two subunits, wherein each of said subunits have not more than one transmembrane domain, wherein said DNA comprises genes for each of said subunits modified by lacking at least a substantial portion of the transmembrane domain and cytoplasmic domain, and comprising at the 3' terminus a signal encoding for lipid attachment in place of the transmembrane domain encoding sequence.

14. A cell according to Claim 13, wherein said signal encoding lipid attachment is joined to said surface membrane protein by a sequence encoding a peptidase cleavage site.

15. A DNA sequence comprising at least two genes, each of said genes encoding a subunit of a poly(subunit) surface membrane protein, wherein at least a portion of each of the transmembrane domains is modified by replacement with a sequence encoding for lipid attachment.

16. A DNA sequence according to Claim 15, wherein said DNA sequence comprises two subunit genes, wherein only one of said genes comprises a transmembrane domain.

17. A DNA sequence according to Claim 15, wherein said two genes encode the subunits of the T cell

receptor.

18. A method of preparing soluble surface membrane proteins, said method comprising:

growing cells according to Claim 10, whereby said surface membrane proteins are produced and bound to the surface by means of lipid attachment; and

cleaving said surface membrane protein from said lipid to release said soluble surface protein from said surface.

19. A method according to Claim 18, wherein said cleaving is with pronase.

20. A method according to Claim 18, wherein said cells are according to Claim 15.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/05082

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

U.S.Cl: 530/350; 435/172.3,240.2,69.7,70.1; 536/27

IPC(5): C07K 13/00; C12N 15/12, 15/62; C07H 21/04

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.Cl.:	530/350; 435/172.3, 240.2, 69.7, 70.1; 536/27

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Online Databases: Automated Patent Searching, 1975-1991; Dialog-Files Medline, Biosis, Chemical Abstracts, World Patents Index

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	Proceedings of the Natioal Academy of Science, Volume 85, issued May 1988, Tykocinska et al., "Glycolipid Reanchoring of T-lymphocyte surface Antigen CD8 using using the 3' End Sequence of Decay-Accelerating Factor's mRNA." pages 3555-3559, see the entire document.	1-5 7-16, 18, 20 1-20
Y	Science, Volume 238, issued 27 November 1987, Caras et al., "Signal for Attachment of a Phospholipid Membrane Anchor in Decay Accelerating Factor," pages 1280-1283, see the entire document.	1-20
Y	Proceedings of the National Academy of Science, Volume 84, issued May 1987, Gascoigne et al., "Secreting a Chimeric T-Cell Receptor Immunoglobulin Protein," pages 2936-2940, see the entire document.	6,17

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
07 November 1991	20 DEC 1991
International Searching Authority	**Signature of Authorized Officer**
ISA/US	R. Keith Baker, Ph.D.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Nature, Volume 317, issued 1985, Becker et al., "Variability and Repertoire size of T-cell receptor V gene Segments," pages 430-434, see the entire document.	6,17
A	Annual Review of Biochemistry, Volume 57, issued 1988, Ferguson et al., "Cell-Surface Anchoring of Proteins via Glycosyl-Phosphatidylinositol Structures," pages 285-320.	1-20